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(54) Title: SOLUBLE T-CELL ANTIGEN RECEPTOR CHIMERIC ANTIGENS		
(57) Abstract Soluble antigens for efficient production of antibodies against human T-cell receptor epitopes are provided. A preferred antigen polypeptide is a chimeric comprising at least 100 amino acids from a V _β domain with solubility conferred by an immunoglobulin heavy chain C _{H2} or C _{H3} domain fused thereto. Product antibodies and methods for diagnostic and therapeutic uses for both antigens and antibodies are provided.		

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SOLUBLE T-CELL ANTIGEN RECEPTOR CHIMERIC ANTIGENS

FIELD OF THE INVENTION

5 This invention relates generally to the construction of fused gene segments using recombinant DNA techniques to produce T-cell receptor chimeric antigens in secretable form and, more particularly, to improved methods for producing antibodies specifically reactive with T-cell receptor epitopes.

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BACKGROUND OF THE INVENTION

The vertebrate immune system is characterized by its ability to respond to an enormously diverse set of antigenic determinants, or epitopes. This response is effected through T and B lymphocytes, commonly referred to as T cells and B cells. The immune system, comprising these specialized cells, recognizes and processes foreign pathogens and macromolecules, typically clearing them from the body. Lymphocytes individually exhibit high specificity in recognition of particular molecular shapes and collectively exhibit great diversity in reacting with, i.e., specifically binding, a broad range of molecular structures. These properties are mediated through both T-cell receptors and immunoglobulins that serve as antigen receptors.

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T-cells usually recognize only antigens that are present on the surfaces of other cells. This recognition event generally occurs only when the antigens are correctly presented in combination with polymorphic cell surface molecules encoded by the major histocompatibility complex (MHC). These T-cells can be subdivided into separate functional categories including cytotoxic effector cells (T_c), inducer or helper cells (T_h) and suppressor cells (T_s).

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35 T-cells mature in the thymus, in contrast to B cells which differentiate in the fetal liver or bone

marrow. Among the cell surface molecules which characterize T-cells are the α and β chains of the T-cell receptor molecule. General introductory review articles describing the T-cell receptor include Toyonaga and Mak, (1987) "Genes of the T-Cell Antigen Receptor in Normal and Malignant T-Cells" in Ann. Rev. of Immunology 5:585-620; Kronenberg et al., (1986) "The Molecular Genetics of the T-Cell Antigen Receptor and T-Cell Antigen Recognition" in Ann. Rev. of Immunology 4:529-591; and Davis, (1985) "Molecular Genetics of the T-Cell Receptor Beta Chain" in Ann. Rev. of Immunology 3:537-560.

T-cell antigen receptor polypeptides are members of the immunoglobulin superfamily. Members of the immunoglobulin superfamily are characterized by similarities in primary and tertiary structure. They also display characteristic gene segment organization, ontogeny of expression and diversification. In particular, the polypeptide chains of the immunoglobulins typically result from the rearrangement, at the genetic level, of various variable region (V), joining region (J), constant region (C) or diversity region (D) gene segments. Although particular chains may have different sized repertoires of the various segments, the genetic rearrangements and combinatorial joining and junctional diversity all contribute in a characteristic manner to produce the extraordinary diversity of polypeptide sequences exhibited by these gene families. See, Hood et al., (1985) Cell 40:225-22.

A T-cell antigen receptor molecule comprises two polypeptide chains, generally an alpha chain and a beta chain, each of which exhibits two extracellular immunoglobulin-like domains. Such molecules comprise an amino terminal variable region domain (V) and a carboxy terminal constant region domain (C), more specifically designated with α or β when indicating the particular chain of origin. Each of these domains is normally stabilized by a disulfide bond between two conserved cysteine residue pairs on each chain. The two chains are

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apparently attached to one another by an inter-chain disulfide bond near the cell membrane outer surface. Each chain is anchored on the membrane by a hydrophobic transmembrane segment which typically spans the entire membrane lipid bilayer. A short carboxy terminal segment extends into the cytoplasm. Both alpha and beta chains are normally N-glycosylated at sites near the disulfide bonds and at a site near the extracellular surface of the cell membrane.

The alpha and beta chains of the T-cell receptor are encoded by gene segments analogous to the variable region (V) segment, the joining region (J) segments, the diversity (D) segment, and the constant region (C) segments of immunoglobulin genes. Diversity in the T-cell receptor repertoire arises, in part, from the rearrangement of V, D, and J gene segments and from the insertion or deletion of nucleotides at the V_{β} -D and D- J_{β} or V_{α} - J_{α} junctions. Some cells express T-cell receptor gamma and delta chains in place of the alpha and beta chains.

Although specimens of alpha and beta chains of T-cell antigen receptors from mouse and from human have been isolated, it has been difficult to raise antibodies of desired specificity against these polypeptides. T-cell receptors are expressed at very low levels on T-lymphocytes, with conventional biochemical purification generally being unfeasible. Thus, physical characterization studies have been almost non-existent. In general, the production of antibodies against native T-cell receptors has also been unproductive.

Previous workers have reported solubilization of the T-cell antigen receptor (TCR) V_{α} domain as a chimeric molecule in either the form V_{α} - C_{H1} -H- C_{H2} - C_{H3} or the form $V_{\alpha}C_{\kappa}$, where C_{H1} -H- C_{H2} - C_{H3} and C_{κ} are, respectively, immunoglobulin heavy and light constant regions. See Gascoigne et al., (1987) Proc. Natl. Acad. Sci. USA 84:2936-2940; Mariuzza and Winter, (1989) J. Biol. Chem. 264:7310-7316). Gascoigne et al. reported solubilizing

the chimeric V_{α} -heavy chain in conjunction with a mouse λ light chain, whereas Mariuzza and Winter reported solubilizing the chimeric $V_{\alpha}C_{\kappa}$ light chain homodimers expressed in the absence of heavy chain expression.

5 Consistently obtaining highly efficient, secreted expression of substantially full length T-cell receptor polypeptide segments remains a major research goal. Although some genetic fusions between T-cell receptor domains and immunoglobulin segments have been
10 constructed, the expression products typically have resulted in insoluble polypeptides. Thus, there exists a need for improved methods for producing large quantities of water-soluble T-cell receptor analogues which will permit the efficient production of antibodies against
15 natural T-cell receptor epitopes. Preferably, when presented to target immune systems the analogues will not induce a significant number of antibodies against epitopes from the non-TCR segments. In particular, polypeptide segments of T-cell receptors which can be
20 effectively reconstituted into conformations highly analogous to their native state would be most preferred.

Furthermore, the absence of a full repertoire of reagents for distinguishing the various T-cell receptor family types has hindered progress in the
25 understanding and treatment of many immunological disorders. The present invention answers these and other needs.

SUMMARY OF THE INVENTION

30 The present invention provides for the efficient production of antibodies, typically monoclonal antibodies (MAbs), to human T-cell antigen receptor (TCR) epitopes. These antibodies have numerous uses as research, diagnostic, and therapeutic tools for studying
35 or treating immune processes mediated by T-lymphocytes. A pre-requisite to the development of these antibodies is the availability of sufficient amounts of immunogen, in

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this case, proteins comprising human TCR epitopes, in highly pure form.

A preferred soluble chimeric polypeptide of the present invention will have at least 100 amino acids from a V_β domain, typically a complete native human V_β domain. The solubility may be conferred by an immunoglobulin heavy chain C_{H2} or C_{H3} domain fused to the V_β domain. Alternatively, a polypeptide comprising $X1-V_{R1}-C_{R2}-X2-C_{H2}-C_{H3}-X3$ can be prepared, wherein

V_{R1} is a variable region domain of a human T-cell receptor chain;

C_{R2} is a constant region domain of a human T-cell receptor chain;

C_{H2} is a second heavy chain constant region domain of an immunoglobulin gene;

C_{H3} is a third heavy chain constant region domain of an immunoglobulin gene;

each of $X1$, $X2$ and $X3$, if present, is a polypeptide segment comprising less than about 50 amino acids; and

$R1$ and $R2$ are each either α or β . $R1$ may be the same as $R2$, but need not do so.

Dimers of two such polypeptides may be employed and may be either homodimers or heterodimers. These polypeptides may further comprise a toxin moiety. The invention further embraces nucleic acids encoding and/or expressing such polypeptides.

A preferred method for producing antibodies capable of binding to a V_α or V_β T-cell receptor domain epitope will comprise the step of exposing to a target immune system a soluble hybrid polypeptide comprising a V_α or V_β T-cell receptor domain. Thereafter, one or more cell lines capable of producing the antibodies can be cloned. Alternatively, the antibodies can be produced by exposing to a target the chimeric polypeptide on a cell surface.

The polypeptides and antibodies of the present invention are useful in various therapeutic regimens and

diagnostic systems. ⁶ Such antibodies may be employed, for instance, to diagnose a disease or disorder by determining specifically what T-cell antigen receptor or antigen molecules are produced, or, if coupled to a toxin moiety, to substantially eliminate a T-cell population. The chimeric polypeptides will be useful in interfering with antigen binding by T-cell receptors characteristic of that population and in efforts to screen for various agents capable of binding to T-cell receptors.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the deletion of CH1 exon from pMV γ 2. Figure 1A is a map of pMV γ 2. E_H is the Ig heavy chain enhancer; C_{H1}, H, C_{H2}, and C_{H3} represent, respectively, the mouse γ 2a heavy chain constant region 1, hinge, constant region 2, and constant region 3 domains. Hyg represents the hygromycin resistance gene. Figure 1B is a schematic description of steps involved in deleting the CH1 exon. The Δ C_{H1} fragment was first ligated with the adapter oligonucleotide shown, further gel purified, and then used in the final ligation step with the vector fragment to generate the construct shown in Figure 1c, which is a map of pMV γ 2(Δ C_{H1}).

Figure 2 is a map of pCMV γ 2(Δ C_{H1}). CMV denotes the ~700 base pair enhancer promoter from human cytomegalovirus.

Figure 3 depicts the deletion of trans-membrane domains from β -TCR. Figure 3A is a hydropathy analysis of predicted amino acid sequence of β -TCR and its alignment with a restriction endonuclease map of the cDNA. Figure 3B shows the deletion of trans-membrane domain from β -chain, and ligation with adapter oligonucleotide for subsequent cloning into Bluescript vector. Nucleotides #33 and #34 of this adapter oligonucleotide are modified from the naturally occurring nucleotides at these positions in the intron so as to introduce an XbaI site.

Figure 4 is a map of the pCMV β 8.1(Δ TM)- γ 2a(Δ CH1) construct.

Figure 5 represents the amino acid sequence from the amino-terminus of the chimeric V β 8.1/mouse γ 2a protein.

Figure 6 depicts quantitative fluorescence histograms of Jurkat cells. Equal amounts of a reference antibody (α V β 8, line 2 in parts A, B, C, & D) and test antibodies were used to stain 5×10^5 Jurkat cells. The staining patterns were visualized by indirect immunofluorescence using flow cytometry on a Becton-Dickinson FACSMate. The staining pattern produced by the specific antibodies are shown by line 3 in each panel. Line 1 in each panel depicts the background staining profile of the cells. The specific test antibodies resulting from immunization of mice with the chimeric β -TCR are: 9B59.10 (Panel A), 9G47.9 (Panel B), 9A15.6 (Panel C), and 9B47.14 (Panel D).

DETAILED DESCRIPTION

The present invention provides novel nucleic acid constructs which, upon expression, produce polypeptides useful for immunizing target immune systems to raise antibodies specifically reactive with native T-cell receptor epitopes. The antibodies exhibit specific binding to native conformation epitopes to provide powerful diagnostic and therapeutic reagents. The present invention also provides generalized techniques for producing fusion polypeptides which are, typically, both soluble and secreted, and possess structural features exhibiting unusually high epitopic similarity to native T-cell receptor molecular structures.

In accordance with one aspect of the present invention, novel gene fusions are provided which comprise a T-cell receptor extracellular domain, such as the variable and constant domains of a T-cell receptor polypeptide chain, and constant region domains of another immunoglobulin superfamily gene, typically a mouse IgG

heavy chain lacking a complete CH1 domain. The resulting gene fusion encodes a hybrid polypeptide than can be secreted by the producing cell in a soluble form, preferably with post-translational modifications characteristic of polypeptides naturally occurring in T-cells. By careful selection of a combination of a desired T-cell receptor V_α or V_β gene and immunoglobulin gene domains, as guided by the rules described herein, the natural conformations of the T-cell receptor domains and the immunoglobulin domains are substantially obtained.

A preferred embodiment of the present invention includes the use of these improved antigens to efficiently produce polyclonal or monoclonal antibodies specific for a selected T-cell receptor molecular structure, in some instances substantially free of any immunological response to the non-TCR epitopes or to unnatural conformational epitopes. The present invention also includes the antigens themselves, their use in structural and functional characterizations, and the uses of both antigens and their reactive antibodies as diagnostic, therapeutic or investigative agents.

General Definitions

In order that the invention may be more completely understood, several definitions are set forth. As used herein, the term "immunoglobulin" refers to a protein substantially encoded by one or more immunoglobulin gene segments. The recognized immunoglobulin genes include κ , λ , α , γ , δ , ϵ , and μ constant region genes, as well as the myriad immunoglobulin variable region genes. See, generally, Hood et al., Immunology, 2nd Ed. (1984) Benjamin Pub. Co., Menlo Park, CA.

The T-cell antigen receptor (TCR) appears to comprise at least two different polypeptide chains in a heterodimer complex. This complex normally contains one alpha polypeptide chain and one beta polypeptide chain.

These two chains are normally held together by an inter-chain disulfide bond. Each of the alpha and beta chains contains one segment designated a variable region (V), and one segment designated a constant region (C).

5 Analogous to the alpha chain, the variable and constant regions of the beta chain are designated V_β and C_β , respectively. In addition to the variable and constant regions, each chain possesses a relatively hydrophobic transmembrane segment and a short intracellular domain.

10 T-cell antigen receptor V genes may also be categorized into categories, either families or subfamilies. Each subfamily can be characterized by a consensus sequence and contains similar gene segments exhibiting greater than about 75% similarity in the DNA
15 sequence. Sixteen mouse V_β sequences, for example, have been categorized into fourteen different subfamilies by a proposed arbitrary but simple numerical nomenclature for the V_β gene segments. See Barth et al., (1985) "The Murine T-cell Receptor Employs a Limited Repertoire of
20 Expressed V_β Gene Segments" Nature 316:517-523. According to this nomenclature, members of the same subfamily share the first digit and differ in second; therefore the $V_{\beta 8.1}$, $V_{\beta 8.2}$ and $V_{\beta 8.3}$ are all members of the $V_{\beta 8}$ subfamily. A similar system has been proposed for the human gene
25 segments, and at least subfamilies $V_{\beta 1}$ - $V_{\beta 20}$ have been identified (see, Toyonaga and Mak, op. cit., at pg. 602).

Similarly, 22 different V_α sequences have been grouped into 11 subfamilies, and Southern blots have shown these families consist of 1 to 10 members each
30 (reviewed in Kronenberg et al., (1986) Ann. Rev. Immunol. 4:529-591). A numerical nomenclature for the mouse V_α gene segment families has been proposed, analogous to that described above for the V_β genes. It is understood that the present invention comprehends additional T-cell
35 antigen receptor variable region subfamilies as they are discovered and characterized.

A "soluble" antigen or polypeptide is characterized by its failure to be sedimented at room

temperature under low G-force centrifugation out of an aqueous buffer. Typically, a protein is considered soluble if the protein, at a temperature greater than about 5°C, at near neutral pH in the presence of low or no concentrations of detergent, has a sedimentation value less than about 10 to 50 svedberg units. Solubility is particularly important in providing simplicity in purification of the hybrid T-cell antigen polypeptide from the producing cell and in presentation of the purified antigen to an immune system to elicit highly specific antibodies. Solubility is also important for use of the fusion polypeptide as a binding receptor for either ligand specific reagents or cognate antigens. Secretion of the hybrid immunogen is also important to maximize ease of purification from producing cells. Aqueous buffers used in protein chemistry typically have a buffering compound for providing a pH, typically within a physiological range of about 5-9, an ionic strength typically between about 10 mM and about 500 mM, often a protease inhibitor and a mild detergent. Often a carrier protein may be added, such as bovine serum albumin (BSA) or chicken ovalbumin to a concentration of a few mg/ml. Examples of standard buffers are phosphate buffered saline, tris-buffered saline or any of a number of buffers used in protein isolation. See generally, volumes of Methods in Enzymology; Mahler and Cordes (1966) Biological Chemistry (2d Ed.) Harper and Row, New York.

A "natural conformation" for a polypeptide domain is that secondary and tertiary conformation of the domain in a hybrid protein exhibiting substantially identical epitopes to a target immune system as the native polypeptide would by itself. Such includes, preferably, antigenic determinants co-translationally or post-translationally supplied, as by glycosylation or modification of amino acid residues or other structural components. Optimally, the tertiary structure of the hybrid polypeptide closely approximates that of a native

homologous segment in its natural state, typically in a natural membrane integrated form. In particular, the spatial relationship and tertiary and macromolecular structure of the hybrid domains should retain virtually identical intra-chain relationships such that the immunological epitopes eliciting antibodies in a target immune system are substantially identical to the structural features of a natural homologous structure. See Watson et al. (1987) Molecular Biology of the Gene, Benjamin Pub. Co., Menlo Park, California.

The term "operably linked" means that a genetic sequence is positioned on the same molecule so as to ensure the function of nucleic acid segments or sequences either upstream or downstream from a given segment. Those nearby segments typically affect processing or expression of the given nucleic acid sequence.

A target immune system is comprised of essential T-cells and B cells as necessary to biologically effect the desired immunological response. Such immune systems will typically be intact animals. Antigens are presented to such animals by injection, with or without appropriate adjuvants. Such immunization should thereby present molecular epitopes to the immune system in a fashion that allows the T-cells and B cells to interact to produce the desired response. Alternatively, such presentation may be performed in vitro under specifically controlled conditions. See, C. Reading, J. of Immunol. Methods (1982) 53:261-291.

In many instances, a target immune system may be substituted by a library of antibody producing cells. See, Huse et al. (1989) "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phase Lambda" Science 246:1275-1281. Thus, a broad repertoire of available gene constructs may be screened to serve either as sources for producing antigens or for antibodies against specific antigens. Recombinant antigens or antibodies may be produced by gene fusion methods described, e.g., in Cabilly et al., U.S.

4,816,567; Boss et al., U.S. 4,816,397 and Winter, EPO 0239400. These references describe sources for the various immunoglobulin domains useful in the fusion proteins of the present invention, along with alternative molecules which may be used as antigens or antibodies.

Finally, in some situations, particularly in production of antibodies, single chain molecules may be substituted, as described in, e.g., Huston et al., Proc. Natl. Acad. Sci. USA 85:423-26 (1988); Bird et al., Science 323:15-16.

An "accessible" cell surface protein is one that, as associated with a cellular structure, is immunogenic when presented to an appropriate target immune system. Thus, a polypeptide whose epitopes are structurally protected from a target immune system is not considered immunologically "accessible".

An "immunotoxin" is a molecule which comprises both a targeting moiety and a toxic moiety. Typically, the targeting moiety is an antibody binding site. The toxic moiety is toxic to the cell or structure to which the targeting moiety attaches. Thus, one form of an immunotoxin is an antibody conjugated to a toxic compound or reagent, such as ricin, providing a functional highly specific targeting of the toxin. Immunotoxins are reviewed in I. Pastan et al., Cell 47:641-648 (1986). Recently, recombinant immunotoxins have been developed (Chaudhary et al., Nature 339:394-399).

Design Considerations

In accordance with the present invention, genetic constructs have been devised to express polypeptide immunogens mimicking T-cell receptor epitopes. These chimeric polypeptides will preferably be readily secreted in soluble form at high expression levels by cells transformed with the genetic constructs.

An exemplary genetic construct comprises a chimeric human T-cell receptor (TCR)/IgG construct domain compound, preferably without a CH1 domain. A chimeric

protein results from the fusion of polypeptide segments originating from different proteins. The resultant protein, also known as a fusion protein, typically exhibits some biological or physical properties of each of the proteins from which the chimera is derived. Thus, fusion proteins of a signal sequence with another protein may result in secretion of an otherwise cytoplasmic protein. Such techniques may provide for proteins exhibiting properties in a single protein characteristic of each individual parent, often from different species. Examples of chimeric proteins include chimeric antibodies providing for molecules exhibiting mouse binding domains and human constant region domains, and fusion between β -galactosidase and other proteins (for determining insertion of cloned segments).

Since hybridoma technology is best understood in the mouse, the preferred embodiment utilizes a human $V_{\alpha}C_{\alpha}$ or $V_{\beta}C_{\beta}$ gene fused with a mouse immunoglobulin gene fragment. This fusion provides that the human TCR portion of the chimeric polypeptide serves as the focus of the immune response when exposed to a target mouse immune system. It is understood that other target immune systems and a corresponding immunoglobulin gene may be substituted for mouse as detailed below.

Because there are inter-chain contact regions between the variable domain and the constant domain of the beta chain of the T-cell receptor, the antigens of a preferred embodiment of the present invention present a more natural conformation between the variable and constant domains of the beta receptor by containing both of these domains as found in a natural beta chain. Molecular modeling studies have been performed on immunoglobulin molecules based upon X-ray crystallographic studies. These studies have indicated the presence of a ball and socket type of contact between amino acid residues in V_H and C_{H1} of the antibody heavy chain. See, Lesk and Chothia, (1988) Nature 335:188-190. These contact residues have been hypothesized as being

important for a conformational change occurring upon antigen binding by the antibody molecule. A homology search for similar residues at equivalent positions in T-cell receptor sequences revealed the presence of such residues in the β -TCR sequence, but not in the α -TCR sequence. Hence in the context of these studies, prior investigators were forcing a very unnatural juxtaposition of dissimilar residues between V_α and C_{H1} by substituting V_α for V_H .

Finally, in the preferred constructs of the present invention, there is no need to produce multiple chains of T-cell receptors, thus eliminating the requirement for coordinated processing between different chains. By appropriate selection of cells for expression, the glycosylation patterns of the alpha or beta chain may be slightly modified. Moreover, it is possible to co-transform a construct containing the alpha chain of the T-cell receptor with another construct containing the beta chain of the T-cell receptor, thereby providing the possibility for association into homodimers of alpha and of beta and heterodimers of alpha with beta.

An exemplary chimeric polypeptide of the present invention is a chimeric human T-cell receptor beta chain/mouse IgG γ 2a heavy chain protein. The preferred genetic construct contains the human β chain 8.1 subtype (YT35), but any other β T-cell type may be substituted. The construct is designed to contain:

L_β - V_β - D_β - J_β - C_β (delta TM)-H- C_{H2} - C_{H3} ,
 where L_β - V_β - D_β - J_β denote one leader peptide, V region, D region and J region of a beta T-cell receptor chain; C_β (delta TM) denotes the constant region of a beta T-cell receptor gene with the transmembrane and intracellular segments deleted (i.e., the 48 carboxy terminal amino acids of the polypeptide); and H- C_{H2} - C_{H3} denote a mouse IgG γ 2a heavy chain hinge, second constant region and third constant regions.

Alternative chimeric constructs of the present invention include combinations of V_α , C_α , V_β , and C_β with

constant region domains from immunoglobulins. Thus, alternative constructions include use of less than full length segments of the TCR domains. Thus, instead of using full length V_β or C_β domains, for example, in the described constructs, smaller segments of the domains may be substituted. These segments will ordinarily be at least about 50 amino acids from the appropriate domains, such as the V_β or C_β domains, preferably at least about 80 amino acids and, in preferred embodiments, up to the full length of the domain.

Alternatively, segments from proteins other than immunoglobulins might be used, and may include proteins from other species. Typically, the non-TCR domains are included for the purpose of conferring either secretion or solubility properties to the chimeric protein. Other useful segments such as ovalbumin, serum albumin, and other soluble proteins might be selected. But typically, immunoglobulin gene family domains will be used, more usually constant domains from immunoglobulin genes, from the mouse or other species. Thus, constant region domains from other species may be substituted.

The fusion proteins also may include between each of the specified domains, either additional domains or spacer segments of polypeptide. Such domains will typically be globular domains which will not interfere with the TCR domains. Spacer segments will typically be selected under similar constraints, but will typically be less than about 200 amino acids, preferably less than about 50 amino acids; more preferably less than about 30 amino acids, and in the preferred embodiment, fewer than 10 amino acids.

As the domains, as described, may be less than the entire domain, the hinge region of the CH_2 domain proximate to the TCR domains may be deleted as well. Likewise, the C_α or C_β domains may be less than the entire domain. In the extreme, particular domains may be totally deleted, for example, one of the constant regions domains may be removed.

5 Additions to the described domains may be included. For example, either immunoglobulin or non-immunoglobulin transmembrane or cytoplasmic domains may be added, providing for expression of the antigen on a cell surface. Typical transmembrane domains include those from the TCR proteins or other cell membrane proteins, from the same or other species.

Besides the expression of single chain proteins, the present invention includes multichain antigens. Among the multichain embodiments are combinations of α chain type fusion proteins with β chain type fusion proteins. For example, simultaneous expression, perhaps coordinate, of $V_{\alpha}C_{\alpha}C_{H2}C_{H3}$ with $V_{\beta}C_{\beta}C_{H2}C_{H3}$ type chains may result in heterodimer formation leading to interchain $V_{\alpha}C_{\alpha}$ domains with $V_{\beta}C_{\beta}$ domains. Alternatively, an IgG type molecule may result from coexpression of $V_{\alpha}C_{\alpha}$ type protein with the fusion protein of $V_{\beta}C_{\beta}$ immunoglobulin domains, or the $V_{\beta}C_{\beta}$ type protein with a fusion protein of $V_{\alpha}C_{\alpha}$ -immunoglobulin domains.

20 The DNA segments will typically further include an expression control DNA sequence operably linked to the chimeric polypeptide coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells and expression therein, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the polypeptides containing the V_{α} or V_{β} epitopes.

35 Human V_{α} or V_{β} domain DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably peripheral T-cells. The immunoglobulins and other domains for producing the chimeric proteins of the present invention

will be similarly derived from B cells. They may be
obtained from any convenient mammalian source, including,
mice, rats, rabbits, or other vertebrate capable of
producing antibodies by well known methods. Suitable
5 source cells for the DNA sequences and host cells for
immunoglobulin and other protein expression and secretion
can be obtained from a number of sources, such as the
American Type Culture Collection ("Catalogue of Cell
Lines and Hybridomas," Fifth edition (1985) Rockville,
10 Maryland, U.S.A.). Immunoglobulin domains have been well
defined and particular sequences may be found in, e.g.,
E. Kabat et al., "Sequences of Proteins of Immunological
Interest" U.S. Dept. Health and Human Services (1983).

In addition to the chimeric polypeptides
15 specifically described herein, other "substantially
homologous" modified immunoglobulins can be readily
designed and manufactured utilizing various recombinant
DNA techniques well known to those skilled in the art.
For example, the C_{H2} and C_{H3} domains can vary from the
20 native sequences at the primary structure level by
several amino acid substitutions, terminal and
intermediate additions and deletions, and the like. In
general, modifications of the genes may be readily
accomplished by a variety of well-known techniques, such
25 as site-directed mutagenesis (see, Gillman and Smith,
Gene 8:81-97 (1979) and Roberts, S. et al, Nature
328:731-734 (1987)).

The nucleic acid sequences of the present
invention capable of ultimately expressing the desired
30 chimeric polypeptides can be formed from a variety of
different polynucleotides (genomic or cDNA, RNA,
synthetic oligonucleotides, etc.) and components (e.g.,
J, D, and C regions), as well as by a variety of
different techniques. Joining appropriate genomic
35 sequences is presently the most common method of
production, but cDNA sequences may also be utilized (see,
European Patent Publication No. 0239400).

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362).

Cell Transformation with the Gene Fusions

Having constructed gene fusions, according to design considerations as described, expression of the antigen products is desired. Appropriate vectors and cell types may be selected which will produce an antigen or reagent which most closely parallels the desired product. For immunogen purposes, it is expected that glycosylation may be one of the primary criteria upon which a cell type selection will be based. Thus, particular cell types may be chosen for their expression properties. In other circumstances, efficiency in secretion or some other cellular property may be more desirable.

Although microorganisms may be used, eucaryotic cells are preferred to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987)). Mammalian cells are actually most preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulin-like proteins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc., but preferably transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen, et al., Immunol. Rev. 89:49-68 (1986)), and necessary processing information

sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the V_{β} domain and $C_{\alpha 2}$ domain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982)).

Once expressed, the chimeric polypeptides of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). Substantially pure polypeptides of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

Antibodies

Once the antigens of the present invention have been constructed and expressed, thereby producing soluble and/or secreted proteins for use as antigens, antibodies

may be produced by presenting the hybrid protein to a target immune system. For production of polyclonal antibodies, an appropriate target immune system is selected. Typically a mouse is used, of the appropriate strain, usually producing heavy chains of the type selected for the fusion domains of the hybrid antigen. The substantially purified antigen is presented to the immune system in a fashion determined by the mouse strain and other parameters well known to immunologists. Typical sites for injection are in the footpads, intramuscularly, intraperitoneally, or intradermally. Of course, different animals may be substituted for a mouse.

Typically, an immunological response is assayed by use of an immunoassay. Normally such immunoassays involve some purification of a source of antigen, for example, produced by the same cells and in the same fashion as the antigen was produced. The immunoassay may be a radioimmunoassay, an enzyme-linked assay, a fluorescent assay, or any of many other choices, most of which are functionally equivalent but may exhibit advantages under specific conditions.

Monoclonal antibodies with affinities of 10^8 M⁻¹ preferably 10^9 to 10^{10} , or stronger will typically be made by standard procedures as described, e.g., in Harlow and Lane (1988), Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory or Goding (1986), Monoclonal Antibodies: Principles and Practice (2d edition) Academic Press, New York. Briefly, appropriate animals will be selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter the cells are clonally separated and the supernatants of each clone are tested for their production of an appropriate antibody specific for the desired region of the antigen. Note that because the heavy chain regions of the hybrid antigen can be selected to be non-immunogenic in the

target immune systems, there is inherently an advantage in the screening of the resultant monoclonal antibody supernatants since clones producing antibodies specific for the immunoglobulin heavy chains should be rare.

5 It will be appreciated by those of skill in the art that the binding regions of the antibody molecules contain critical amino acid residues for contact and binding in epitope recognition. Thus, these complementarity determining regions (CDR's) which produce
10 specificity of binding are the structural features conferring specificity in binding. For the binding functions of these antibodies, molecules which exhibit sufficiently similar spatial relationship of these CDR's will be functionally equivalent molecules. Gene fusions
15 or deletions leading to molecules which provide CDR's based on antibodies of the present invention will obviously serve, in many cases, to provide equivalent functions.

20 Selecting Specific V_{β} Antibodies

 One important objective of this invention is the development of an array of antibodies capable of distinguishing the various forms of the T-cell receptor molecules. Thus, it is desirable to produce antibodies
25 analogous to anti-idiotypic antibodies of immunoglobulin genes.

 A large array of antigens might be produced from different V_{β} chains against which each of a large number of different T-cell receptor specific antibodies
30 might be tested. By comparing which antibodies are specific for a single or few subfamilies or specific V_{β} chains, those antibodies will become diagnostic for particular receptor subfamilies or chains. Antibodies which show broad specificity for most or all subfamilies
35 find different uses in diagnostic or therapeutic contexts as described below.

Diagnostic, Therapeutic and Investigative Utilities

The present invention provides means for the efficient and economical production of antibodies directed to each of the various T-cell receptor chains. By providing the means for producing enormous amounts of a soluble and secreted antigen which elicits, specifically, only antibodies reactive with the desired structural domains, the immunization of target immune systems is dramatically simplified. This allows the generation of a large repertoire of antibodies specific for binding to individual members of T-cell types, families, subfamilies or combinations.

The hybrid polypeptides of the invention are also useful in structural and functional studies on the T-cell receptor. Besides serving as substrates or binding domains of specific forms of receptor, these analogues may serve in conformational studies which approximate the native configurations as subjects. Such studies provide approximate structural or functional data which might be easily verified with smaller amounts of native TCR chains.

The solubilized chimeric receptors also serve as binding competitors to block normal cellular binding of particular ligands, thus serving to clear the ligand or to compete with the physiological binding by cells. The effective amount of such a receptor sufficient to interfere with the binding of an antigen to a T-cell antigen receptor will depend, for instance, upon the particular affinity of the receptor for the ligand in question. A likely effective dose of receptor to be administered is between 100 μ g to 100 mg daily for a human subject. Thus, these antigens which exhibit binding domains with the native protein binding specificity may be used in a fashion equivalent to standard antibodies for certain purposes. Qualitative and quantitative assays for ligand binding are well known to those skilled in the art. See, for instance, E.

Harlow and D. Lane, (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor).

These antigens may also be used to simulate the native binding domain to investigate what ligand may serve as an agonist or antagonist for the selected fusion polypeptide. Thus, the binding domain is used as a screen to select those ligands which will bind to the encoded alpha or beta chain variable region.

With the antibodies of the present invention, a repertoire of antibodies may be generated which can easily distinguish the particular type, family or subfamily of a given cell. Thus, if a clonally overproduced cell type is produced, as may occur in, for example, a leukemia condition, it is possible to determine the type of cell involved, and then readily produce a targeted immunotoxin for substantially eliminating those cells. By substantially eliminating, it is meant that the cell number is reduced by 50%, preferably 80%, most preferably 90% or more.

Determination of the cell type can also serve as a diagnostic tool for the purpose of investigation into the problems of, or the treatment of a condition. The use of antibodies raised against T-cell receptors have been successful in treating experimental allergic encephalomyelitis (EAE). See, e.g., Acha-Orbea et al. (1988) Cell 54:263-273; and Urban et al. (1988) Cell 54:577-592.

In addition, the antibodies or binding proteins of the present invention are useful as idiotypic antibodies to determine specifically what T-cell receptor or antigen molecules are expressed within an organism. Such may be important in, among other disorders or diseases, T-cell malignancies, autoimmune diseases, transplant/graft rejection reactions, host versus graft disease, cancers, solid tumors, allergies and various infectious diseases. Quantitative or qualitative measurement of types of expressed T-cell antigen receptors allow diagnosis and monitoring of a patient

with multiple infections or a disease involving a plurality of responses. It is understood that both qualitative and quantitative measurements are embraced by the present invention. By use of the term diagnosis is also meant the monitoring of the severity or progression of a disease or disorder or the recovery of a subject from a disease or disorder.

Accordingly, patient samples are tested for expression of characteristic T-cell receptors. Test samples may comprise biological fluids including but not limited to blood, plasma, serum, saliva, urine, spinal fluid, synovial fluid, amniotic fluid and cranial fluid. Quantitation may be performed by comparison to known standard samples.

The following examples are offered by way of illustration, not by limitation.

EXPERIMENTAL

Vector

1. Deletion of C_{H1} exon from the expression vector.

The antibody domains chosen for incorporation into the chimeric β -TCR construct were derived from the vector pMV γ 2. This vector was itself derived by replacing the XbaI-BamHI fragment of PV γ 1 (Queen et al. (1989), Proc. Natl. Acad. Sci. USA 86:10029-10033) containing the human constant region with an XbaI-BglIII fragment containing the mouse γ 2a constant region, a ClaI linker being used to connect the BamHI and BglIII ends. A map of pMV γ 2 along with pertinent structural features and relevant restriction sites is presented in Fig. 1A. The vector contains the full mouse gamma 2a constant region downstream of a unique XbaI cloning site. The first modification of the vector involved deletion of the C_{H1} exon. This was accomplished as outlined in Fig. 1B. Specifically, 50 μ g of pMV γ 2 were digested with XbaI and ClaI to produce a 6.7 kb "vector" fragment, and a 4.1 kb fragment containing the full mouse gamma 2a constant region. These fragments were recovered by electroelution

following fractionation on a preparative 0.9% agarose gel. In order to delete the C_{H1} exon from the γ 2a fragment, the 4.1 kb XbaI/ClaI fragment was subsequently digested with StuI. StuI cuts in the intron between the C_{H1} and Hinge (H) exons, 47 nucleotides downstream of the intron donor site. Thus, the purified 4.1 kb γ 2a fragment was further digested with StuI, and the resultant 1.6 kb XbaI/StuI "C_{H1} fragment" and 2.3 kb StuI/ClaI fragments recovered following a second round of agarose gel fractionation and electroelution. The 2.3 kb StuI/ClaI fragment, which contains the Hinge (H), and second and third heavy chain constant region exons (C_{H2}, C_{H3}), will be henceforth referred to as the Δ C_{H1} fragment. The Δ C_{H1} fragment was ligated with the following synthetic adapter oligonucleotide to restore an XbaI site at its 5' end:

5' CTAGAGGTTCACAAGTGATTAGTCTAAGG 3'
3' TCCAAGTGTTCACTAATCAGATTCC 5'

the sequence of this adapter oligonucleotide is derived from the naturally occurring sequence in the intron between the C_{H1} and H exons for 28 of the 29 nucleotides upstream of the StuI site in this intron. The first nucleotide of this oligonucleotide, shown underlined, was modified from the naturally occurring nucleotides so as to reconstitute the desired XbaI site. The naturally occurring nucleotide at this position in the intron is a G. The first three nucleotides of the hexanucleotide StuI site are highlighted in boldface.

The adapter ligated 2.3 kb Δ C_{H1} fragment was purified away from unligated excess adapter by fractionation on an agarose gel and recovered by electroelution. Finally, this was religated to the 6.7 kb XbaI/ClaI "vector" fragment from step 1 above to reconstitute a Δ C_{H1} version of the original vector, referred to as pMV γ 2(Δ C_{H1}), shown in Fig. 1C. Preparative amounts of this first modification of the vector were

obtained by transformation of E.coli DH1 competent cells with the products of ligation of the 6.7 kb "vector" fragment and the 2.3 kb adapter ligated ΔC_{H1} fragment. Transformed colonies were positively identified by their Amp^r phenotype, and the ΔC_{H1} modified vector was purified in preparative quantities using conventional plasmid purification procedures (Sambrook et al., (1989) in Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Laboratory). The sequence of the artificial junction between the XbaI and StuI sites was verified by DNA sequence analysis of the plasmid.

2. Replacement of Immunoglobulin heavy chain promoter/kappa enhancer with the human cytomegalovirus enhancer/promoter.

Comparative studies carried out at Protein Design Labs on relative strengths of promoter enhancer combinations leading to highest levels of expression of a desired gene product have established the human cytomegalovirus (HCMV) enhancer/promoter as superior to the immunoglobulin heavy chain enhancer/ κ promoter combination for the production of humanized antibodies in myeloma cells. Since we wished to achieve the highest possible levels of expression of the chimeric V β 8.1/mouse γ 2a protein, we further engineered the pMV γ 2(ΔC_{H1}) vector by replacing the immunoglobulin heavy chain enhancer/ κ promoter with the HCMV enhancer/promoter.

The HCMV enhancer/promoter was isolated as an approximately 600 bp FnuD2 fragment extending from position -522 to position 72 (numbered according to Boshart et al. (1985) Cell 41:521-530), and an EcoRI linker was added at the 5' end and an XbaI linker at the 3' end.

The deletion of the immunoglobulin enhancer/promoter from pMV γ 2(ΔC_{H1}) was effected by digestion of 8 μ g of pMV γ 2(ΔC_{H1}) with XbaI and HindIII. This digest generated a 4.5 kb fragment containing the γ 2(ΔC_{H1}) and Hygromycin^r genes (referred to as fragment A)

27

and a 4.4 kb fragment containing the Ig promoter/ κ enhancer, pBR322 ori sequences, and a SV40 polyadenylation fragment (referred to as fragment B). Following size fractionation by agarose gel electrophoresis and recovery by electroelution, fragment A was set aside for later use, and fragment B was subjected to secondary digestion with EcoRI. This produced three fragments: a 2.64 kb EcoRI/HindIII fragment containing pBR322 ori sequences, and the SV40 fragment polyadenylation signal; a 1.13 kb EcoRI/XbaI fragment containing the Ig heavy chain promoter; and a 0.7 kb EcoRI fragment encoding the κ enhancer. The 2.64 kb EcoRI/HindIII fragment was recovered by electroelution following agarose gel fractionation, and treated with alkaline phosphatase from calf-intestine to prevent self ligation and polymerization in subsequent steps.

The 4.5 kb XbaI/HindIII fragment (fragment A), 2.6 kb phosphatased EcoRI/HindIII pBR322 ori, SV40 fragment, and 600 bp EcoRI/XbaI HCMV enhancer/promoter encoding fragments were combined in a triple ligation experiment to produce the desired vector, designated pCMV γ 2(ΔC_{III}), shown in Fig. 2. This vector was recovered by transformation of competent *E. coli* with an aliquot of the triple ligation reaction mixture. Potential colonies containing the desired vector construct were selected on the basis of their Amp^r phenotype, and plasmid mini-preps from Amp^r colonies were purified and analyzed by diagnostic restriction endonuclease digests to identify those colonies containing the correct vector construct, as predicted from the map in Fig. 2.

3. Deletion of the Trans-membrane Domain from the β -TCR constant region.

A full length cDNA insert containing a functionally rearranged human V β 8.1 T-cell receptor sequence was obtained from the clone YT35 (Yanagai et al., (1984) *Nature* 308:143-149) as an XbaI/SphI fragment,

and transferred into a Bluescript vector (Stratagene) and designated pBS β 8.1.

Hydropathy analysis (Chou and Fasman, (1978) Adv. in Enzymol. 47:45-147) of the predicted amino acid sequence encoded by this cDNA serves to identify a putative membrane spanning region at the carboxy terminal end of this protein. A unique StuI site is situated just upstream of the trans-membrane (TM) and cytoplasmic domains of the β T-cell receptor, and hence, serves to delineate them from the extra-cellular V,D,J, and C domains (Fig. 3 A). Analytical digests of pBS β 8.1 plasmid isolated from DH1 strain of E.coli, with StuI showed the plasmid refractory to digestion with this enzyme. This was discovered to be due to a methylation modification of a cytosine residue contained within the StuI site by dcm methylase, which rendered the site non-cleavable by StuI (New England Biolabs, 1988-1989 product catalog, p.143). Hence, the plasmid pBS β 8.1 was transferred into E.coli HB101, which is dcm modification negative. Thus, 112 μ g of pBS β 8.1, isolated from HB101 transformed cells, were digested with XbaI and StuI. The 817 bp β -TCR insert deleted of its trans-membrane domain (Δ TM) was recovered by electroelution after fractionation in 1% agarose gel.

The purified $\beta(\Delta$ TM) fragment was ligated with a synthetic 37-mer oligonucleotide, as outlined in Fig. 3B, to restore the StuI site abutting the fragment, and reintroduce an XbaI site at its 3' end. The first 15 nucleotides of this oligo were derived from the C β sequence to preserve the β -TCR residues immediately extracellular to the plasma membrane. The remaining 22 nucleotides of this oligo-nucleotide are derived from the intron donor site and the first 16 nucleotides downstream of it, in the intron between the C β and H exons from the vector pMV γ 2. Nucleotides #33 and #34 of this adapter oligonucleotide are modified from the naturally occurring nucleotides at these positions in the intron so as to introduce an XbaI site, to facilitate cloning of the

transmembrane deleted insert fragment into the (ΔC_{H1}) modified vector (see Vector, above, and Fig. 4 below).

The adapter ligated β -TCR(ΔTM) fragment was purified from unligated adapter oligo by fractionation on an agarose gel and recovered by electroelution. It was then introduced into the XbaI site of the Bluescript vector (Stratagene) for amplification by preparative isolation of plasmid from transformed bacteria. This plasmid was designated pBS $\beta 8.1(\Delta TM)$.

Generation and Verification of the Final Construct

Generation of the final construct is outlined below, and a map of the construct is presented in Fig. 4. For generation of the final construct, the vector pCMV $\gamma 2(\Delta C_{H1})$ was linearized by digestion with XbaI, followed by phosphatasing of 5' termini with calf intestinal alkaline phosphatase. Meanwhile, the ~880 bp $\beta 8.1(\Delta TM)$ fragment was purified by electroelution of XbaI digested pBS $\beta 8.1(\Delta TM)$ fractionated on an agarose gel. The $\beta 8.1(\Delta TM)$ insert was then ligated into the XbaI linearized and phosphatased vector and the products of the ligation reaction used to transform competent E. coli.

Transformed colonies were selected on the basis of resistance to ampicillin, and plasmids analyzed by diagnostic restriction digestion of "mini-prep" DNAs purified from small scale cultures. The mini-prep DNAs were digested with XbaI to verify presence of an insert fragment of the expected size (880 bp). Finally, orientation of insert relative to promoter was determined by digestion of the DNAs with StuI. Those clones with insert in the same orientation as the promoter were chosen for final sequence verification of relevant portions of the molecule. The results from DNA sequence analysis of the constructs at the 5' end of the insert and at the 3' insert/vector junction containing the artificial splice donor site, across the XbaI and StuI sites, and into the C_{H1} -Hinge intron, agreed exactly with

the expected sequences at these junctions³⁰, and verified the correctness of the the construct. Following sequence confirmation of the identity of the constructs, the CMV enhancer/promoter construct was designated pCMV β 8.1(Δ TM)- γ 2a(Δ C_{H1}).
5

Expression of Chimeric T-Cell Receptor/Immunoglobulin Protein

1. Transfection of Cells and Detection of Expressing Cell Lines.
10

For expression of the CMV enhancer/promoter driven chimeric V β 8.1 T-cell receptor/immunoglobulin heavy chain construct, the construct [pCMV β 8.12(Δ TM)- γ 2a(Δ C_{H1})] was linearized with BamHI. The linearized DNA
15 was extracted with phenol and chloroform, ethanol precipitated, and resuspended in 1X PBS at a concentration of 1 μ g/ μ l (measured by A₂₆₀). The constructs were transfected by electroporation into two myeloma cell lines, Sp2/0, and 25-1. Sp2/0 is a B-cell
20 tumor line which does not secrete endogenous immunoglobulins, while 25-1 is an Sp2/0 derived cell line generated at Protein Design Labs, Inc., Mountain View, CA, which secretes high levels of a humanized anti-TAC κ -light chain as Bence-Jones homodimers (Queen et al.,
25 (1989) Proc. Natl. Acad. Sci. USA 86:10029-10033).

Hence, 10⁷ cells in 1 ml PBS from each of these lines were transfected by electroporation (Chu et al., (1987) Nucl. Acids Res. 15:1311-1326) with 20 μ g of the BamHI linearized construct from above using a Bio-Rad
30 Gene Pulser, and transformed cells were selected on the basis of resistance to hygromycin. Hygromycin selection was carried out on a daily basis, starting 24 hr after transfection, by feeding the cells with media supplemented fresh daily with 700 μ g/ml (\pm 10%)
35 hygromycin. This daily feeding was continued for 10-12 days, after which surviving cells were allowed to reach confluence in the 96-well micro-titre plating dishes. Cells were expanded in selective media by passage from

the 96-well plates into 24-well plates, and supernatant aliquots were assayed for presence of chimeric β -TCR/ γ 2a protein by ELISA assay once the culture reached confluency in 24-well plates.

5 The ELISA assay for detection of the chimeric β -TCR protein was carried out as follows: ELISA plates (Immulon) were coated with 200X dilution of goat anti-mouse IgG (Heavy + Light chain, Tago Immunochemicals) in 1X PBS at 4°C for overnight to several days. Following
10 blocking of non-specific protein binding sites in the plate with 1% Bovine Serum Albumin (BSA), 0.1% Tween-20, 0.01% sodium azide in 1X PBS, for at least 2 hr at 4°C, the plates were incubated with test antigens (supernatants from confluent 24-well plate cultures of
15 hygromycin resistant transfected cell lines), in duplicate. The plates were incubated with antigen for 2 hr at room temperature, and then washed 3X with 200 μ l/well of 1X phosphate buffered saline (PBS)/0.1% TWEEN-20. The plates were then incubated for 1 hr at
20 room temperature with 200X dilution of horseradish peroxidase (HRP) conjugated rabbit anti-mouse Ig γ 2a (Zymed Immunochemicals) in 1X PBS as the detection reagent. Positive cell lines were identified by virtue of a green color reaction upon addition of HRP substrate
25 (Biorad).

Table 1 below summarizes the number of expressing primary cell lines obtained from transfection of Sp2/0 and 25-1 cells with the construct pCMV β 8.1(Δ TM)- γ 2a(Δ CH1).

30 Table 1. Ratio of expressing cell lines transfected with Construct

	<u>Cell Line</u>	<u>pCMVβ8.12(ΔTM)-γ2a(ΔCH1)</u>
	Sp2/0	4/10
35	25-1	32/32

32

The results show that the frequency of expression of the chimeric protein was significantly higher in 25-1 cells (>95%) than in naive Sp2/0 cells (~35%).

5 This difference in frequency of expression between 25-1 and Sp2/0 cells was paralleled by the level of expression of the chimeric β -chain (as measured by ELISA assay using mouse anti-TAC as a concentration standard). The highest level of expression observed from the Sp2/0 primary transfectants was 90 ng/ml for the CMV enhancer/promoter driven construct. By contrast, the
10 highest level of expression observed in 25-1 primary transfectants was 200 ng/ml for the CMV enhancer promoter driven construct. Thus, comparing cell lines, 25-1 was superior to Sp2/0 in both the frequency of expression as well as the overall level of expression.
15

In summary, the data presented above from ELISA assays of supernatants from primary transfectants establish the following three facts: (1) the frequency of expression from transfection of the chimeric β -TCR
20 construct is greater in 25-1 cells than in Sp2/0 cells; (2) the overall level of expression attained is greater from 25-1 cells than from Sp2/0 cells; and (3) the transfectants observed are secreting a protein which contains mouse immunoglobulin- γ 2a determinants.
25

2. Immunochemical Characterization of the Secreted Protein.

The ELISA data presented above established, at a preliminary level, the secreted expression of a protein
30 containing mouse immunoglobulin- γ 2a determinants, as evidenced by the immuno-crossreactivity with the HRP-conjugated 2° reagent in the assay. In order to further characterize the biochemical properties of the secreted molecule, and thereby more firmly establish its identity,
35 we analyzed the secreted protein by SDS-PAGE following immunoprecipitation of metabolically labelled proteins from ELISA positive cell lines.

For this analysis, two ELISA positive cell lines were selected at random, and cells arising from the primary transfection were expanded into 10 ml cultures for metabolic labelling of proteins by incubation with ³⁵S-methionine. The metabolic labelling and subsequent immunoprecipitation were carried out essentially as described in Harlow and Lane, (1988) in Antibodies, A Laboratory Manual, Cold Spring Harbor, New York. Briefly, one confluent plate each of cells ($0.5-1 \times 10^7$ cells) from experimental (transfected) and control (untransfected) lines were harvested by centrifugation at 1000 rpm in a benchtop clinical centrifuge, per labelling reaction. The cell pellets were washed 2X with sterile 1X PBS (37°C), 1X with methionine free media (37°C), and then resuspended in 1 ml each of methionine free media (Dulbecos Modified Eagle Medium, without L-methionine, without L-glutamine, GIBCO) supplemented with dialyzed 10% fetal calf serum (FCS, Hyclone), and 2 mM glutamine. The cells were incubated at 37°C for 30-45 min to deplete endogenous methionine pools. Protein labelling was then carried out by addition of 100-200 µCi of ³⁵S-methionine (Amersham, $\geq 1,000$ Ci/mmol), and a further incubation of the cultures at 37°C for 5-7 h. The metabolically labelled cells were harvested as described above, and the supernatant fractions saved for analysis of secreted proteins by immunoprecipitation as described below. In order to analyze intracellular synthesis, the cells were lysed by resuspending the cell pellets in 0.9 ml ice cold Tris buffered saline (TBS, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl), and lysed immediately by addition of 5% nonidet-P40 (NP40) to 0.5% final concentration. The protease inhibitor, phenylmethanesulfonyl fluoride (PMSF) was added to a final concentration of 1 mM, and the lysate(s) incubated on ice for 20 min. Cellular debris/insoluble material was removed from the cell lysate by centrifugation at $12,000 \times g$ for 10 min at 4°C. The supernatant from this step, containing cell lysate, was recovered and saved for further analysis.

Preliminary immunoprecipitations with this crude lysate and supernatant fractions revealed a high level of background, presumably from non-specific adsorption of metabolically labelled proteins to the antibody conjugated agarose beads employed in the experiment. Hence, these two fractions were routinely pre-cleared by overnight adsorption to a rabbit IgG-agarose beads (Sigma, 100 μ l beads/ml lysate or supernatant), which served as a non-specific adsorbent. The cleared supernatants were recovered following a brief centrifugation to remove the non-specific adsorbent, and used in subsequent immunoprecipitation experiments described below.

Immunoprecipitations of radiolabelled protein were carried with 100 μ l aliquots from pre-cleared cell lysates and supernatants prepared as described above. The samples were immunoprecipitated directly with both rabbit IgG-agarose and protein-A agarose, using 5 μ l protein conjugated agarose resin/100 μ l sample, at 4°C for 1-3 hr with constant agitation using a TOMY micro tube mixer. Alternatively, the samples were also immunoprecipitated in a two step procedure using a purified 1° antibody (1 μ l/100 μ l sample) at 0 - 4°C for 1-3 hrs, followed by protein A-agarose as described above. The samples were then prepared for washing by a brief centrifugation at 12,000g for 15 - 30 seconds. The radioactive supernatant was removed by aspiration. The immunocomplex coated agarose beads were washed 3X with 1 ml/wash of a solution containing 0.5% NP-40, 0.2% sodium deoxycholate, and 0.1% SDS in 1X TBS (NDS solution), followed by a final wash with 1X TBS (to remove detergents). The immune complex coated agarose beads were air dried overnight at room temperature prior to SDS-PAGE analysis of the immunoprecipitated product.

For polyacrylamide gel analysis of the immunoprecipitated products, the samples were resolubilized in Laemmli sample buffer (2% SDS, 10% glycerol, 10 mM DTT (dithiothreitol), and 60 mM Tris-Cl

pH 6.8) in a boiling H₂O bath for 10 min. For analysis on non-reducing gels, the samples were resolubilized in Laemmli buffer minus DTT. Aliquots of the resolubilized samples were then loaded onto 10% or 12% polyacrylamide gels containing 0.1% SDS prepared and run according to the method of Laemmli, (1970) Nature 277:680-688 described in Harlow and Lane, (1988) in Antibodies, A Laboratory Manual, Cold Spring Harbor, New York. Two-dimensional gel analysis of the samples was carried out essentially as described in O'Farrell (1975) J. Biol. Chem. 250:4007-4021. Analytical experiments using broad pI range test IEF gels established the pI of the immunoprecipitated protein was in the neutral pH range. Consequent fine pI range titration further established a pH range in the IEF gels of 6-8 as optimal for maximal resolution of the proteins isoforms. Hence, the isoelectric focusing gels contained 2% (vol/vol) ampholytes pH range 4-10, and 4% (vol/vol) pH 6-8 ampholytes (BioRad). Following electrophoresis, gels were fluorographed, dried, and the results visualized by autoradiography.

Immunoprecipitation of cell lysates or supernatants with protein-A agarose revealed the exclusive presence of a major polypeptide band of 68 kD apparent molecular weight in both the intracellular and supernatant fractions of material from transfected cells, and its complete absence from untransfected cells. A protein of identical molecular weight is also immunoprecipitated with a two step procedure using either affinity purified goat anti-mouse IgG γ (G α mIg γ) as the 1^o antibody followed by protein A-agarose, or goat anti-human κ (G α Hu κ) as the 1^o antibody followed by protein A-agarose.

Hence, the immunoprecipitation data confirmed the secreted expression by transfected cells only (25-1, as well as Sp2/0), of a protein bearing mouse IgG γ 2a determinants. The data furthermore extend the observations from ELISA assay and document the

intracellular expression³⁶ of this protein by transfected cells only. The apparent molecular weight of this protein, 68 kD, agrees favorably with its predicted molecular weight, 54 kD, based on knowledge of its predicted primary structure. The 14 kD difference can be accounted for by post-translational modifications, e.g. glycosylation, which are known to occur on antibody molecules. A major site for N-linked glycosylation is in the CH2 domain of the molecule, which is retained in our chimeric construct.

A second major conclusion from the one-dimensional SDS-PAGE analysis of immunoprecipitated products is that the putative chimeric β -TCR/ γ 2a protein, a chimeric Ig heavy chain, is expressed in the absence of any requirement for assembly with light chain prior to its secretion from the cell. This is evidenced by two observations. The first observation is that immunoprecipitation of radiolabelled proteins from transfected 25-1 cells by either protein A-agarose alone or with G α m γ followed by protein A-agarose failed to co-immunoprecipitate a polypeptide in the known size range of Ig light chains (~25 kD). The cell line 25-1 was derived from Sp2/0 by transfecting it with a humanized anti-TAC κ light chain construct. Thus, it is expressing high levels of a humanized κ light chain. The second, and more conclusive piece of evidence in support of the notion that the chimeric β -TCR/ γ 2a heavy chain is secreted in the absence of any requirement of prior assembly with a light chain, is its expression in Sp2/0 cells. Sp2/0 cells do not secrete any endogenous light chain, yet this protein is immunoprecipitated from both lysate and supernatants of transfected cells only, by either protein A-agarose alone, or G α m γ followed by protein A-agarose.

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3. The Putative Chimeric ³⁷ β -TCR/ γ 2a Heavy Chain is Secreted as a Homodimer.

The hinge domain in antibody molecules contains two important cysteine residues which are responsible for inter-chain disulfide linkages between immunoglobulin heavy chains. The formation of these interchain disulfide bonds is important to the structural integrity of native antibody molecules. We were interested in determining whether these sites would retain their native properties, i.e. serving to cross-link heavy chain monomers into dimers by disulfide linkages, in the chimeric molecules encoded by our construct. Thus, in order to obtain information regarding the quaternary structure of the protein, we analyzed its electrophoretic properties under non-reducing conditions.

For this analysis, radiolabelled proteins from transfected and untransfected cells were immunoprecipitated as described. Subsequent to drying, the immunoprecipitated products were resolubilized in Laemmli buffer lacking the reducing agent DTT. The solubilized immunoprecipitated products were analyzed by SDS-PAGE on 6% polyacrylamide gels. The pattern of immunoprecipitated products is similar to that observed earlier. Both protein A-agarose alone, and GamIg γ followed by protein A-agarose, specifically immunoprecipitate a readily detectable product from transfected cell lysates and supernatants. A major difference, however, between the results observed in the absence of reducing agent compared with those in the presence of reducing agent is in the apparent molecular weight of the immunoprecipitated products. The apparent molecular weight of the immunoprecipitated product under reducing conditions (where both intra- and inter-chain disulfide bonds are reduced) was 68 kD. Under non-reducing conditions, the mobility of this protein in the supernatant fraction, shifts to approximately 110 kD.

Immunoprecipitation of the intracellular fraction revealed a doublet of bands, both of higher

apparent molecular weight ³⁸ than observed under reducing conditions. One of these two bands is of similar apparent molecular weight as that observed in the supernatant fraction. The second band is -96 kD apparent molecular weight. One explanation, given that it is present only in the intracellular fraction of transfected cells, is that it corresponds to an unglycosylated form of the putative chimeric β -TCR heavy chain. The plausibility of this explanation arises from two facts. First is that the sites for N-linked glycosylation in the CH2 domain are contained within the chimeric molecule. Second, glycosylation was demonstrated to occur and indeed was required for the secreted expression of a chimeric $V_{\alpha}C_{\kappa}$ light chain Mariuzza and Winter, (1989) J. Biol. Chem. 264:7310-7316. Thus, the apparent molecular weight of the non-reduced, presumably unglycosylated protein, is approximately double its predicted molecular weight of 54 kD derived from its primary structure.

Therefore, the data from analysis of immunoprecipitated products under non-reducing conditions established that the chimeric TCR/heavy chain is cross-linked via disulfide linkages to form a homodimer. Although we do not have direct evidence, the most probable site for this is cross-linking is via the cysteine residues encoded in the hinge domain of the Ig portion of the molecule.

4. The Chimeric Protein Secreted from Cells Transfected with pV β 8.1(Δ tm)- γ 2a(Δ C_{H1}) Contains β -TCR Determinants.

A commercially available antibody that cross-reacts with common determinants on human β -TCR chains, β F1 (T-Cell Sciences), was used to demonstrate, by western blot analysis, the presence of human β -TCR determinants on the chimeric protein secreted by transfected cells. For this analysis, a 25-1 primary cell line expressing the putative chimeric β -TCR/heavy chain protein was cloned by limiting dilution. Cell lines expressing higher levels of the chimeric protein,

as assayed by the ELISA method described above, were identified. One of these was expanded further and used as a source for preparative isolation of chimeric β -TCR from large scale tissue culture supernatants.

5 For purification of preparative amounts of the protein, the clonal cell line was first acclimatized to growth under serum-free media conditions. Acclimatization to serum free media conditions was carried out initially on a gradual basis over the course
10 of approximately 7-10 days. A dense 10 ml culture of cells ($\sim 10^6$ cells) growing in DME-High Glucose media (Irvine Scientific) supplemented with 10% FCS was passaged daily by splitting the plate 1->2, and fed with DME-High Glucose media supplemented with 1% Nutridoma NS
15 (Boehringer Mannheim), such that the vol/vol ratio of the final media after feeding increased from 9.0% FCS supplemented media/0.10% Nutridoma supplemented media on day one following the initial split, to 0% FCS supplemented media/1% Nutridoma supplemented media at the
20 end. Once a cell culture had been established and reached a vigorous growth state in serum free media, the culture was expanded to preparative scale for isolation of the chimeric β -TCR. Initially, 0.5 - 0.6 liter cell cultures were employed as starting material. The cells
25 were maintained in 1.0 l. flasks as 100 ml cultures for 2-5 days, and then harvested. Secreted proteins in the supernatants were then concentrated using an Amicon concentrator (model no 8200), and either PM10, PM30, or XM50 filtration membranes. The starting supernatants
30 were concentrated to 15-20 ml final volume. The chimeric β -TCR protein was purified from this concentrated supernatant by a one-step affinity purification on a recombinant protein-A column (Repligen) essentially as described in Harlow and Lane, (1988) in Antibodies, A
35 Laboratory Manual, Cold Spring Harbor, New York. Briefly, binding of the ligand to the column was achieved by two passages of the concentrate over the column, and the column was washed with 10 volumes of 1X PBS. The

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ligand was eluted from the column with 5 volumes of 50 mM Glycine (pH 2.5), 150 mM NaCl. The eluant was collected in 1 or 2 ml fractions (depending on the volume of the column), and the pH immediately raised to neutral by addition of a pre-titrated amount of 1 M Tris-Cl (pH 9.0). The protein peak in the eluant was identified by A_{280} measurement, which also served as an initial estimation of the yield of total protein.

The presence of β -TCR determinants in the putative chimeric protein were established by western blot analysis. For this experiment, 1-2 μ g of total protein eluted from the protein-A column was loaded, following denaturation in reducing Laemmli buffer, onto a preparative well (1 mm x 8 mm x 4 mm), and electrophoresed into a 12% polyacrylamide gel. The proteins were then electrophoretically transferred onto nitrocellulose membrane for 2-3 hr with a Transphor model TE-50 electroblotter (Hoeffer Scientific Instruments) as described in Harlow and Lane, (1988) in Antibodies, A Laboratory Manual, Cold Spring Harbor, New York. Following transfer, the membrane was marked to assign polarity with respect to the original gel, and then mounted in a PR150 mighty small deca probe western blot staining apparatus (Hoeffer Scientific Instruments), and incubated in 2-3 ml/lane of 3% BSA, 1X PBS for 1 hr at room temperature to block non-specific protein binding sites on the membrane.

The presence of β -TCR determinants in the affinity purified secreted protein from transfected cells was documented using a three-step reagent system. The reagents were: 1*-100X diluted β F1 MAb (overnight); 2*-biotin conjugated goat anti-mouse kappa (Fisher Biotech) affinity purified antibody (1-2 hr); and 3*-1000 X diluted alkaline phosphatase conjugated streptavidin (Sigma, 1/2-1 hr). All reagents were diluted in 1% BSA, 1X TBS, 0.1% NaN₃, and the blot washed between addition of subsequent reagents with 1X TBS, 0.1% Tween-20. Following the 3* reagent, the blot was washed with 1X

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TBS, and then incubated with alkaline phosphatase substrate (Nitro Blue Tetrazolium + 5-Bromo-4-chloro-3-indoyl-phosphate, Sigma, as described in Harlow and Lane, Supra) to visualize antibody-antigen complex. In control experiments, it was established that the 3° reagent alone did not cross react with the test antigen when diluted at least 100X, and the 2° reagent plus 3° reagents alone did not cross react when the 2° reagent was diluted at least 1000X.

The results of this experiment established that at a 1000X (or higher) dilution of the 2° reagent, an immuno-crossreactive product is observed only when the blot was incubated with the 1° reagent, β F1. Furthermore, the apparent molecular weight of this product, 68 kD, agrees precisely with that of the protein-A immunoprecipitated products described above. Therefore, this data established the presence of β -TCR determinants in the secreted product expressed by the transfected cell lines.

The purity of these affinity purified protein preparations was assessed by quantitative one and two-dimensional SDS-PAGE, and by these criteria, typical purity of the chimeric β -TCR from batch to batch varied between 95-99%. The major contaminant between batches was usually the humanized κ -light chain (migrating at ~20 kD apparent molecular weight), which is also expressed by the transfected cell line used for the purification of the chimeric β -TCR.

The purity of chimeric β -TCR batches was assessed by silver-staining of one-dimensional SDS-PAGE. Silver staining was carried out using a kit from Stratagene, and the instruction provided by the manufacturer. This analysis revealed the presence of a major polypeptide band of 68 kD apparent molecular weight as the primary elution product from the protein-A column. This corresponds precisely with the size of the polypeptide immunoprecipitated by protein-A agarose, as well as the β F1 immuno-cross reactive product, in the

experiments described above.⁴² (A faint band constituting 1-5% of the total yield was detectable in the 20 kD size range. This corresponded to the humanized κ light chain). This data established that a 68 kD protein is the predominant constituent in affinity purified protein from supernatant derived from transfected cells, and in conjunction with the evidence described above, this molecule bears both T-cell receptor, and immunoglobulin heavy chain determinants.

A more critical assessment of the purity of a protein preparation is its electrophoretic profile on two-dimensional SDS-gels (Harlow and Lane, (1988) in Antibodies, A Laboratory Manual, Cold Spring Harbor, New York). Thus, in order to further characterize the protein heterogeneity of the 68 kD polypeptide band observed in the one dimensional gels, we analyzed the affinity purified protein preparation by two dimensional SDS-PAGE. In the interest of brevity, the results of this analysis will only be summarized here, as they simply confirmed the conclusions discussed above. Two-dimensional analysis of the affinity purified chimeric Vb β .1/mouse γ 2a protein revealed it to be comprised of at least six isoforms, all of which had the same apparent molecular weight, and differed in their isoelectric points. The pI of the chimeric protein isoforms ranged from pH 7-7.5. Furthermore, all the isoforms of the affinity purified chimeric protein also possessed β TCR determinants by virtue of their cross-reactivity to the β F1 antibody in a western blot analysis.

We conclude, therefore, that all the isoelectric variants observed in the affinity purified chimeric protein contain both immunoglobulin heavy chain determinants, as well as β -TCR determinants. Possible contributors to the iso-electric heterogeneity of immunoglobulin molecules which have been documented include post-translational modifications, e.g. glycosylation (Williamson, (1978) in Handbook of Experimental Immunology (D.M. Weir, ed.), pp 9.1-9.31,

Blackwell Scientific Publishers, Oxford, England). This is consistent with evidence presented earlier regarding the difference between predicted and apparent molecular weight of the chimeric protein, and the observation of a lower molecular weight form exclusively in the intracellular fraction when analyzed by non-reducing SDS-PAGE.

5. Evidence That The Chimeric β -TCR/Heavy Chain Protein is V β 8.1 in its T-cell receptor domain.

The immuno-crossreactive data described above provide very convincing evidence that the protein secreted from cells transfected with the pV β 8.1 construct contains both β T-cell receptor determinants, and immunoglobulin heavy chain determinants, as expected. In order to obtain direct evidence of this fact, and to provide final verification of the identity of the chimeric protein as V β 8.1 specific, we subjected the purified protein to N-terminal amino acid sequence analysis.

Accordingly, 18 μ g/-260 pmoles of affinity purified chimeric protein, isolated as described above, were concentrated further by overnight precipitation at 4°C in 5% trichloroacetic Acid (TCA). The precipitated protein was concentrated by centrifugation at 12,000 X g, washed 2X with 90% ethanol, and dried in a speed-vac concentrator. The pellet was subjected to amino acid sequence analysis. The results from the sequenator are summarized in Fig. 5, which shows a comparison of the predicted amino acid sequence of V β 8.1 deduced from the cDNA sequence, and the amino-terminal sequence determined from the affinity purified chimeric β -TCR protein. Amino acid residues whose identity could not be ascribed with 100% confidence are indicated in parentheses in Fig. 5. The sequence of the purified protein agrees exactly with the predicted sequence of V β 8.1 for the first 10 residues sequenced (residues #2-11), and for 12 of the 14 residues sequenced. The identity of the first residue, gly,

although in agreement with the predicted⁴⁴ sequence, cannot be ascribed with confidence due to a technical limitation.

5 The yield per residue at each cycle of the sequenator is a critical parameter in protein sequence determination. The magnitude of this parameter is useful in ascribing the degree of confidence to the sequence obtained as originating from the major/sole polypeptide in the preparation as opposed to originating from a minor
10 contaminant in the protein preparation (due to, e.g., a blocked amino-terminus of the major constituent). Typically this value is highest for the first few cycles, and drops at each progressive cycle thereafter, for technical reasons. The yield per residue for the first
15 two amino acids determined (V and I), was 122 pmoles, and 130 pmoles, respectively. Based on the estimated initial amount of protein submitted for sequence analysis, 260 pmoles, this represents a yield per residue of 45-50%. Hence, we can be confident that the sequence determined
20 was from the major polypeptide in the preparation, as opposed to a minor contaminant in the preparation.

 The results from sequence analysis show that the amino-terminus of the purified protein expressed by the transfected cells agrees precisely for the first 10
25 residues, with that of V β 8.1, starting one residue downstream of the predicted leader peptide cleavage site. This evidence establishes two important facts. First, the identity of the chimeric β -TCR/immunoglobulin heavy chain can be assigned positively and unambiguously as
30 V β 8.1 in its T-cell receptor domain. Second, it shows that the β -TCR leader peptide is correctly processed in myeloma cells, a heterologous cell line.

 Therefore, we conclude that myeloma cells transfected with pCMV β 8.1(Δ TM)- γ 2a(Δ CH1) express a
35 soluble protein which is a chimeric V β 8.1/mouse γ 2a molecule into the extracellular media.

6. Production of Monoclonal Antibodies Targeted to Native β -TCR determinants from Mice Immunized with Solubilized Chimeric V β 8.1/mouse γ 2a Protein.

5 Preliminary experiments carried out to test the feasibility of using the solubilized chimeric β -TCR for generating antibodies to native determinants indicated that the solubilized form would be an effective molecule for this purpose. In the discussion below, the native form of the TCR refers to the α - and or β -chain as it occurs on the surface of T-lymphocytes in association with the CD3 complex.

10 Immunization regimens were initiated in mice to determine the optimal immunization strategy which would lead to the desired antibodies in the shortest time period using the least amount of antigen. Two basic parameters were investigated in this regard: the adjuvant (complete Freund's versus RIBI (RIBI Immunochemical, Hamilton, MT)), and the immunization route (intraperitoneal versus footpad).

20 The immunocompetence of the mice for fusion was assessed by monitoring serum antibody levels via two assays. The serum titre levels were determined by the ELISA assay described above using chimeric β -TCR coated plates, and the quality of the immune response was measured by the ability of immune sera to immunoprecipitate β -TCR specifically from cell surface radio-iodinated proteins (Marchalonis, (1969) Biochem. J. 113:299-305) from Jurkat cells. Radio-iodinated cell surface proteins from MOLT4 cells, which do not express TCR/CD3 on their cell surface, served as negative controls in this experiment. Immunoprecipitations were carried out using 5 μ l of immune sera (the antibody) from mice (diluted 100X in 1X PBS) plus 10⁶ c.p.m. of I¹²⁵-labeled lysate (the antigen) from Jurkat versus MOLT4 cells, in a total volume of 50-100 μ l. The antibody/antigen mixtures were incubated at 4-8°C for ~90 min. The immune complex was precipitated using 5 μ l of goat-anti mouse IgG (Sigma) with agitation for a further

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60 min at 4°C. The immunoprecipitates were washed as described earlier, and analyzed by one-dimensional SDS-PAGE. Serum from non-immunized mice served as a negative control, and rabbit IgG-agarose was the non-specific immunoadsorbent. The quantity of antigen used for immunization was determined to not be a critical factor. Thus, it typically ranged between 5µg - 50µg in the immunizations described below.

It was determined that all immunizations led to a detectable immune response against the solubilized chimeric β -TCR as measured by the ELISA assay. However, only serum titre levels in excess of about 1:2,000 had antibodies which were also immunocompetent in the immunoprecipitation assay. Intraperitoneal immunization with Freund's adjuvant required a 3-4 month interval between primary immunization and first boost in order to reliably and reproducibly elicit these strong titres. Two to four week rest periods between primary immunizations and subsequent boosts via this route using Freund's adjuvant led to highly variable immune responses. By contrast, RIBI adjuvant proved superior to Freund's in consistently eliciting strong immune responses (serum titres \geq 1:5,000 + immunoprecipitation competent sera) in a shorter period between the primary immunization and first boost (2-4 weeks) when the mice were immunized intraperitoneally.

Immunization via the footpad route (Holmdahl et al., (1985) J. Imm. Methods 83:379-384; Mirza et al., (1987) J. Imm. Methods 105:235-243), using RIBI adjuvant, proved to be of superior utility with regard to the two important parameters of quantity of antigen required and the length of immunization necessary. For footpad immunizations, popliteal lymph nodes served as the donor organ of lymphocytes for fusion with a myeloma fusion partner. With respect to the time factor, the fusions were carried out 11 days following the primary immunization. The experiment yielded an abundance of independently derived hybridoma lines (n=32) which were

secreting antibodies targeted against CD3 exposed determinants on the TCR β -chain. Hence, as detailed below, this immunization regimen proved to be superior to all others tested for the generation of anti-TCR antibodies, while requiring the least amount of antigen. The details of this, and the identification of the relevant hybridoma lines are presented below.

Four mice were immunized with $\sim 6\mu\text{g}$ of antigen in $50\mu\text{l}$ RIBI adjuvant in each of their rear footpads following anesthesia. The degree of the anesthesia was monitored by the loss of the leg withdrawal reflex upon a pinch stimulus of the footpad (mice were not injected with antigen until this reflex had completely disappeared). Following immunization, the mice were monitored on a daily basis for the next four to seven days for signs of motor impairment as a consequence of the immunization. They exhibited no overt signs of impairment or physical discomfort. The mice were killed 11 days subsequent to the single immunization, and their popliteal lymph nodes were dissected for fusion with the myeloma fusion partner P3X63Ag8.653 (ATCC CRL1580). Fusion of lymphocytes with myelomas was carried out essentially as described in Harlowe et al. (Harlow and Lane (1988), in Antibodies, a Laboratory Manual, Cold Spring Harbor, NY), using the method of fusion by stirring with the modifications noted below: The ratio of lymphocyte:myeloma employed was 1:2, and the cells were plated following fusion at a density of 1×10^5 lymphocytes in $50\mu\text{l}$ HAT media per well (onto a 24 hr old feeder layer of 2×10^4 peritoneal exudate derived mouse macrophage cells per well), in 96-well plates. The peritoneal exudate was obtained as described (Fazekas DeSt.Groth and Scheidegger, (1980) J. Imm. Methods 35:1-21). Following harvesting, the exudates were washed 1X in HAT media (DMEM, 20%FCS, pen and strep, 1X OPI, 1X HAT, $5 \times 10^{-5}\text{M}$ β -mercaptoethanol, 5mM L-glutamine), and resuspended to the appropriate density for plating in $50\mu\text{l}$ HAT media per well. Fused hybridoma cells were left

undisturbed at 37°C in 7.5% CO₂ atmosphere for seven days following plating.

The cells were fed with 100 µl/well HAT media on day 8. The spent media was aspirated off and the
5 cells were fed 200 µl/well fresh HAT media on day 12. By day 14, individual wells showing active hybridoma growth were easily discernable by visual examination, and were screened as soon as the media in individual wells just began turning yellow. Screening consisted of a two step
10 procedure, a preliminary ELISA assay to rapidly identify anti-β TCR antibody secreting lines, followed by a flow cytometric analysis using indirect immunofluorescence to identify that subset of anti-β TCR antibody secreting lines whose antibodies were targeted to CD3 exposed
15 determinants of the β-chain. Those lines which tested negative in the initial ELISA assay were discarded.

The ELISA assay was carried out using chimeric β-TCR coated plates as the capture agent. Specifically, ELISA plates were coated with 100 µl/well of affinity
20 purified chimeric Vβ8.1/mouse γ2α protein at a concentration of 1.5 µg/ml (diluted in 1X PBS) for at least 24 hr at 4°C. The plates were then blocked for at least 24 hr with 200 µl/well of ELISA buffer. Following washing with 1X PBS, 0.1% Tween-20, test antibodies (i.e.
25 supernatants from HAT resistant hybridoma cells) were applied to the wells and the plates incubated at room temperature for at least 2 hours. Next, the plates were incubated with 100 µl/well of 2000X diluted, biotin conjugated GαMIgκ (Fisher Biotech) for 1 hr at room
30 temperature. Finally, the plates were incubated with a 3° reagent, 200X - 500X diluted horseradish peroxidase conjugated strepavidin (Sigma) at room temperature for 30 - 60 min. The plates were washed in between reagents with three changes of 1X PBS, 0.1% Tween-20, using 200
35 µl/well. Following the 3° reagent, the plates were washed 4X with 1X PBS alone. Positive samples were visualized by application of HRP substrate (BioRad). Hybridoma lines were screened using typically 100µl of supernatant

from the wells in which they were growing in the 96-well microtitre plates. Those lines which tested positive out of the 96-well plates were expanded to 24-well plates (1 ml cultures), whereupon they were retested as soon as

5 their media again just began to turn yellow. In this second round of screening, triplicate aliquots of supernatant were removed from the wells for retesting by the ELISA assay, as well as for their ability to stain Jurkat cells and MOLT4 cells by flow cytometry. For cell

10 staining, 5×10^5 Jurkat or MOLT4 cells (in 50 - 100 μ l 1X PBS, 0.01% sodium azide) were incubated with hybridoma supernatant at 4°C for 30 min. The cells were washed 1X with 250 μ l/well PBS/azide, and incubated with 25 μ l/well FITC conjugated GaMIgG for another 30 min as above.

15 Following a final wash, the cells were fixed by resuspending in 50-200 μ l 1% paraformaldehyde, and analyzed by flow cytometry on a Becton-Dickinson FACS scan. The level of background/non-specific staining (control) was established using media alone, and positive

20 controls for staining intensity included the following three antibodies: OKT3 (Ortho Diagnostics), WT31 (sold as TCR-1 Becton-Dickinson, Cambridge, MA), α V β 8 (T-Cell Sciences). The specificity of these antibodies is targeted to the CD3 complex on T-lymphocytes (Kung et al, 25 (1980) Proc. Natl. Acad. Sci. USA 77:4914-4917), a shared determinant between TCR α / β and the CD3 complex (Van der Griend et al., (1988) J. Immunology 140:1107-1110), and the Jurak β -TCR chain (Tian, et al., (1989) FASEB J. 3:A486 Abstracts), respectively.

30 Those lines which tested positive by the ELISA assay, positive for Jurkat staining and negative for MOLT4 staining were expanded further into 3 ml cultures. Only two out of 34 lines which showed positive cell surface staining profiles on Jurkat cells also showed

35 weakly positive profiles on MOLT4 cells. These cell lines were retested a third time, This time by flow cytometry alone. At this stage of their expansion, in addition to the Jurkat and mMOLT4 lines, all positive

hybridoma lines were tested⁵⁰ against three additional TCR negative T-lymphoma cell lines, namely MOLT3, CEM and HuT102. With the exception of the two lines which had tested positive against MOLT4 cells, the remaining 32 lines again were negative in their staining profiles on these additional T-cell lines. Those lines which tested Jurkat+/MOLT- a second time were expanded further into 10 ml cultures, and the lines were either frozen away or cloned immediately by limiting dilution. The results of the numbers from the fusion are summarized in Table 2 below.

Table 2: Summary of results from fusion of lymph node derived lymphocytes with a Myeloma cell line for the generation of hybridomas producing antibodies targeted to native determinants of the β -TCR chain.

	<u>Description</u>	<u>Number</u>
	Total wells plated	672
	No wells plated	467
20	No ELISA positive	214
	No Jurak (+)/MOLT4(-)	32
	No Jurak (+)/MOLT4(+)	2

In addition to the ELISA and cell staining criteria for identifying hybridoma lines described above, the identity of the antigen which the antibodies produced by these lines were targeted against was further confirmed by their ability to immunoprecipitate β -TCR. Supernatants from ELISA+/Jurkat+/MOLT4- hybridoma lines were used to immunoprecipitate cell surface radio-iodinated proteins from Jurkat and MOLT4 lines. The results revealed all hybridomas tested immunoprecipitated the TCR β -chain from Jurkat cell lysates.

All hybridoma lines which passed each of these criteria were either frozen for later analysis or cloned immediately for further characterization. Seven lines were selected for cloning by limiting dilution for

further characterization. Subsequent to cloning of positive cell lines, four lines were chosen on the basis of their Jurkat staining profiles for antibody purification. For this purpose, clonal lines were grown under serum free media culture conditions and expanded to 100 ml stage. The cultures were maintained at this level until $\geq 75\%$ of the culture consisted of dead cells. At this time, the culture was harvested and the supernatant was concentrated to ~20 ml in an Amicon concentrator using a P10 membrane. Antibody was purified from the concentrate using FPLC based protein-A affinity chromatography. As the four lines selected to purify antibody from were secreting heavy chains of the $\gamma 1$ isotype, the salt was adjusted to 1M NH_4SO_4 in order to facilitate binding of the antibody to the column. Purity of the antibody preparations was verified by SDS-PAGE. Silver stain visualization of the acrylamide gels revealed homogeneous preparation of antibody in all four cases, consisting of just a heavy chain and a light chain band, with no minor contaminants detectable. Antibody yields were quantitated by A_{280} measurements, and by quantitative ELISA assay using a goat anti-mouse IgG coated plate. Quantitative FACS analyses were carried out using precisely matched amounts of the reference antibody ($\alpha\text{V}\beta 8$) and the four purified antibodies. Results of the FACS analysis using the purified antibodies to stain Jurkat cells are shown in Fig. 6. The results show that three of the four antibodies produce histograms identical to the reference antibody $\alpha\text{V}\beta 8$, while the fourth antibody, 9B47, yields a slightly brighter level of staining than the reference antibody. This feature is a consistent hallmark of this antibody, in that it was observed at every level of analysis from initial identification through cloning, and ultimately with the purified antibody itself. The brighter staining observed with this antibody indicates that 9B47 is targeted to a different epitope than the reference antibody.

Thus, the solubilized chimeric β -TCR/IgG heavy chain molecule is a completely native molecule, particularly with respect to the TCR domains, on the basis that antibodies raised against the soluble form cross react with native form of the receptor. As such, it represents a proven and viable antigen for generating antibodies targeted to native determinants of any given member of the TCR family.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.

WHAT IS CLAIMED IS:

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1. A composition comprising a substantially pure soluble chimeric polypeptide having at least 100 amino acids from a V_β domain and at least about 50 contiguous amino acids from an immunoglobulin superfamily protein.
2. A composition of Claim 1, wherein the polypeptide comprises a complete native V_β domain.
3. A composition of Claim 1, wherein the V_β is a human V_β .
4. A composition of Claim 1, wherein the solubility is conferred by an immunoglobulin heavy chain CH2 or CH3 fused to the V_β domain.
5. A substantially pure chimeric polypeptide comprising $X1-V_{R1}-C_{R2}-X2-C_{H2}-C_{H3}-X3$, wherein
- V_{R1} is a variable region domain of a human T-cell receptor chain;
- C_{R2} is a constant region domain of a human T-cell receptor chain;
- C_{H2} is a second heavy chain constant region domain of an immunoglobulin gene;
- C_{H3} is a third heavy chain constant region domain of an immunoglobulin gene;
- each of X1, X2 and X3, if present, is a polypeptide segment comprising less than about 50 amino acids; and
- R1 and R2 are each either α or β .
6. A chimeric polypeptide of Claim 5, wherein the variable region domains and constant region domains are selected from the group of $V_{\alpha 1}$ to $V_{\alpha 11}$ or the group of $V_{\beta 1}$ to $V_{\beta 20}$.

7. A chimeric polypeptide of Claim 5, wherein at least one of the heavy chain constant regions is a mouse heavy chain constant region.
- 5 8. A chimeric polypeptide of Claim 5, wherein the chimeric polypeptide is soluble in an aqueous buffer.
9. A chimeric polypeptide of Claim 5, wherein the V_β is the V_β 8.1 T-cell receptor chain.
- 10 10. A chimeric polypeptide of Claim 5, further comprising a toxin moiety.
11. A chimeric polypeptide of Claim 10, wherein the toxin moiety is covalently linked to the chimeric polypeptide.
- 15 12. A soluble polypeptide comprising human V_α - C_α or V_β - C_β domains fused to mouse immunoglobulin constant region, wherein the mouse immunoglobulin constant region has an incomplete CH1 domain.
- 20 13. A dimer of two polypeptides, wherein each polypeptide is a chimeric polypeptide of Claim 5.
- 25 14. A nucleic acid encoding a polypeptide of Claim 1, 5 or 12.
15. A composition comprising a nucleic acid encoding a polypeptide of Claim 1, 5 or 12, said nucleic acid operably linked to a heterologous promoter or enhancer region.
- 30 16. A method for producing antibodies capable of binding to a V_α or V_β T-cell receptor domain epitope comprising the step of exposing to a target immune system a soluble hybrid polypeptide comprising a V_α or V_β T-cell receptor domain.
- 35

17. A method of Claim 16, wherein the V_{α} or V_{β} domain is a human V_{α} or V_{β} domain.
- 5 18. A method according to Claim 16, further comprising the step of cloning one or more cell lines capable of producing the antibodies.
- 10 19. A method for producing antibodies capable of binding to a human V_{α} or V_{β} T-cell receptor domain epitope, said method comprising the step of exposing to a target immune system a chimeric polypeptide of Claim 5 or a V_{α} or V_{β} chimeric polypeptide on a cell surface.
- 15 20. A method according to Claim 19, further comprising the step of cloning cells secreting antibodies reactive with the human V_{α} or V_{β} domain epitope.
- 20 21. An antibody produced by a method of Claim 16.
22. An antibody of Claim 21 which is a monoclonal antibody.
- 25 23. A method of diagnosing a disease or disorder of a mammal comprising the steps of:
mixing an antibody of Claim 21 with a sample from the mammal; and
detrmining whether the antibody binds to the sample, thereby determining whether a T-cell antigen
30 receptor or antigen molecule is produced in the mammal.
- 35 24. A method of treating a mammal comprising administering to the mammal an effective amount of a polypeptide of Claim 5 sufficient to interfere with the binding of an antigen to a T-cell antigen receptor characteristic of a T-cell.

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25. A method of treating a mammal comprising administering to the mammal an amount of a polypeptide of Claim 10 effective to substantially eliminate a T-cell.

5 26. A method of Claims 24 or 25, wherein the mammal clonally overproduces the T-cell.

27. A method of Claims 23, 24, or 25, wherein the mammal is a human.

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28. A method of screening for an agent capable of binding a T-cell antigen receptor, comprising the steps of:

15 adding a polypeptide of Claim 5 to a sample containing the agent; and
determining the amount of the agent bound to the polypeptide.

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